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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,397,376**, on August 9, 2002, by **OTTAWA HEALTH RESEARCH INSTITUTE**, assignee of May Griffith, for "Innervated Artificial Tissues and Uses Thereof".

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INNERVATED ARTIFICIAL TISSUES AND USES THEREOF

ABSTRACT

The present invention provides an *in vitro* three dimensional, innervated artificial tissue comprising a bio-synthetic matrix which supports cell growth, including epithelial and endothelial surface coverage and three-dimensional cell in-growth, as well as nerve in-growth. The present invention also provides methods of preparing the innervated artificial tissue and methods of innervating artificial tissues or tissue substitutes. The present invention further provides for the use of the artificial tissue for *in vitro* testing of various pharmaceutical, cosmetic and household products.

FIELD OF THE INVENTION

The present invention pertains to the field of tissue engineering and in particular to an innervated artificial tissue for *in vitro* testing applications.

BACKGROUND

The three-dimensional culture of cells create *in vitro* tissue-based systems that closely mimic the natural extracellular matrix is desirable in the development of suitable alternatives to animals for toxicological and irritancy testing.

International Patent Application No. PCT/CA99/00057 describes an artificial cornea constructed by layered growth of different cell lines. In general, however, the three dimensional growth of cells is based sytems that employ hydrogels. U.S. Patent No. 6,103,528, for example, describes the use of a thermally reversible gelling co-polymer for *in vitro* cell culture in three dimensional matrices. Cells are suspended in an aqueous solution of the hydrogel precursor, and then become entrapped within the synthetic matrix upon polymerisation.

In order to function effectively as tissue substitutes, however, three dimensional cell cultures require functional nerve in-growth. A need still exists therefore, for a innervated artificial tissue as a suitable alternative to animal testing.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

An object of the present invention is to provide an innervated artificial tissue and uses thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the general structure of the terpolymer of N-isopropylacrylamide, (NiPAAm), acrylic acid (AAc) and N-acryloxysuccinimide (ASI).

Figure 2 depicts (A) Transparent TE cornea with surrounding ring of opaque collagen (•). Bar, 1 cm. DRG were either placed within the cornea or in the surrounding collagen ring. In either case, bundles of neurites course through the corneal stroma (B) to reach the targeted epithelium. Bar, 30 μm. Inset shows corresponding deep stromal nerves seen by *in vivo* confocal microscopy within the human cornea. From the stroma, nerves branch to form a sub-epithelial plexus in both the fabricated cornea (C) and human cornea (inset). Bar, 20 μm. (D) Smooth (arrowhead) and single beaded nerve fibres (arrow) that migrate into the epithelium of the TE corneas as previously reported in humans (17). Bar, 25 μm. (E) TEM showing a nerve (arrow) invaginating an epithelial cell in the TE cornea. Mitochondria (m), vesicles (arrowhead) and microtubules can be seen. Bar, 0.5 μm. (F) Higher TEM magnification of a nerve fibre penetrating an epithelial cell in the TE cornea, with dense (white arrowheads) and clear vesicles (black arrowheads). Bar, 0.4 μm.

Figure 3 depicts nerve fibres growing into a TE cornea from the scleral scaffold, double labelled with an anti-neurofilament antibody marker for nerve fibres (A) and for sodium channels (B). Bar, 15 μm. (C) Example of a raw, unsubtracted trace evoked by a constant voltage stimulus pulse delivered to the ganglion cell cluster, illustrating a large stimulus artefact, with a small action potential occurring near the top (arrow). (D) Subtraction of the response obtained after lidocaine application isolates the compound action potential (arrow), with short latency and an amplitude of about 26 μV.

Figure 4 depicts (A) Normalized total healing (change in wound area (mm^2)/ original wound circumference (mm)) for TE corneas with and without DRG. At 6, 12 and 18 hours there is significantly faster wound healing for corneas with DRG. Corneas with DRG and controls, n = 16. *$P < 0.05$ versus control (2-way ANOVA). (B) Epithelial cell proliferation in wounded corneas with and without innervation. The percentage of BrdU-positive epithelial cells was significantly greater in innervated corneas compared to non-innervated controls at 0, 6 and 24

hours. The percentage of BrdU-positive cells within each treatment group did not increase over time post-wounding, indicating that epithelial cell proliferation did not increase in the first 24 hours post-injury. All groups, n = 3. *P < 0.05 versus controls (2-way ANOVA). (C) SP release over time from innervated corneas treated with 1% capsaicin versus vehicle-treated controls. The release of SP was significantly greater at 1, 3, 6, 12 and 24 hours in capsaicin treated corneas compared to controls. All groups, n = 3. *P < 0.001 versus control (2-way ANOVA). (D) The normalized SP release from innervated corneas treated with 1% capsaicin or 50 μM veratridine versus controls at various time intervals post-treatment. A significant increase in SP release was observed at 6 and 24 hours post-treatment for capsaicin and after 24 hours for veratridine treatments compared to controls. All groups, n = 6. *P < 0.05 versus control (3-way ANOVA).

Figure 5 depicts Innervated TE cornea (A) and a non-innervated control (B) treated with detergent and stained with live/dead stain (ethidium bromide and acridine orange). Red indicates dead cells; green indicates live cells. Bar, 50 μm. (C) Whole mount confocal microscopic image of nerves (arrowheads) and blood vessel-like structures (arrow) in the fabricated pseudo-sclera surrounding a TE cornea. Bar, 20 μm. (D) *In vitro* nerve growth patterns within the collagen-poly (N-isopropyl polyacrylamide) composite as viewed by confocal microscopy. Surface neurites are labelled red, and neurites inside the polymer, labelled green and blue are at depths of 5 μm and 15 μm, respectively. Bar, 20 μm.

Figure 6 depicts a three-dimensional endothelial cell culture system.

Figure 7 depicts an innervated cornea and sclera model.

Figure 8 presents the results from zymographic detection of metalloproteases.

Figure 9 presents the effects of growth factors on angiogenesis.

Figure 10 presents the effects of Retinol acetate on angiogenesis.

DETAILED DESCRIPTION OF THE INVENTION

It should be understood that this invention is not limited to the particular process steps and materials disclosed herein, but is extended to equivalents thereof as would be recognised by those ordinarily skilled in the relevant arts. It should also be understood that terminology employed herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

The term "hydrogel" as used herein refers to a cross-linked polymeric material which exhibits the ability to swell in water and to retain a significant portion of water within its structure without dissolving.

The term "polymer" as used herein refers to a molecule consisting of individual monomers joined together. In the context of the present invention, a polymer may comprise monomers that are joined "end-to-end" to form a linear molecule, or may comprise monomers that are joined together to form a branched structure.

The term "monomer" as used herein refers to a simple organic molecule which is capable of forming a long chain either alone or in combination with other similar organic molecules to yield a polymer.

The term "co-polymer" as used herein refers to a polymer that comprises two or more different monomers. Co-polymers can be regular, random, block or grafted. A regular co-polymer refers to a co-polymer in which the monomers repeat in a regular pattern (*e.g.* for monomers A and B, a random co-polymer may have a sequence: ABABABAB). A random co-polymer is a co-polymer in which the different monomers are arranged randomly or statistically in each individual polymer molecule (*e.g.* for monomers A and B, a random co-polymer may have a sequence: AABABBABBBAAB). In contrast, a block co-polymer is a co-polymer in which the different

monomers are separated into discrete regions within each individual polymer molecule (*e.g.* for monomers A and B, a block co-polymer may have a sequence: AAABBBAAABBB). A grafted co-polymer refers to a co-polymer which is made by linking a polymer or polymers of one type to a another polymer molecule of a different composition.

The term "bio-polymer" as used herein refers to a naturally occurring polymer. Naturally occurring polymers include, but are not limited to, proteins and carbohydrates.

The term "synthetic polymer" as used herein refers to a polymer that is not naturally occurring and that is produced by chemical or recombinant synthesis.

The term "bioactive agent" as used herein refers to a molecule or compound promotes or facilitates cell growth on or into a natural or artificial tissue. Representative examples include growth factors, retinoids, cell adhesion factors, laminin, hormones, osteogenic factors, and active fragments thereof.

INNERVATED ARTIFICIAL TISSUES

The present invention provides innervated artificial tissues based on bio-synthetic matrix scaffolds which support cell and nerve growth.

I. Bio-Synthetic Matrix

A bio-synthetic matrix according to the present invention comprises a synthetic polymer and a bio-polymer. The matrix is capable of supporting nerve in-growth and cell growth, including epithelial and endothelial surface coverage (*i.e.* two dimensional, 2D, growth) and three-dimensional (3D) cell in-growth. The matrix can further comprise one or more bioactive agents such as growth factors, retinoids, cell adhesion factors, laminin, and the like. The bioactive agent can be covalently attached to the synthetic polymer, or it may be encapsulated and dispersed within the final matrix. The matrix may also comprise cells encapsulated and dispersed therein, which are capable of proliferating upon exposure to appropriate culture conditions.

1.1 Synthetic Polymer

In accordance with the present invention, the synthetic polymer that is incorporated into the bio-synthetic matrix comprises one or more of an acrylamide derivative, a hydrophilic co-monomer and a derivatised carboxylic acid co-monomer which comprises pendant cross-linking moieties.

As used herein, an "acrylamide derivative" refers to a N,N'-alkyl substituted acrylamide or methacrylamide. Examples of acrylamide derivatives suitable for use in the synthetic polymer of the present invention include, but are not limited to, N-isopropylacrylamide (NiPAAm), N,N'-diethylacrylamide, N-acryloylpyrrolidine, N-ethylacrylamide, N-isopropylmethacrylamide, N,N'-diethylmethacrylamide, N-methacryloylpyrrolidine, N-ethylmethacrylamide, and combinations thereof.

A "hydrophilic co-monomer" in the context of the present invention is a hydrophilic monomer that is capable of co-polymerisation with the acrylamide derivative component of the synthetic polymer. Examples of suitable hydrophilic co-monomers are hydrophilic acryl- or methacryl-compounds such as carboxylic acids, acrylamide, methacrylamide, hydrophilic acrylamide derivatives, hydrophilic methacrylamide derivatives, hydrophilic acrylic acid esters and hydrophilic methacrylic acid esters. The carboxylic acid may be, for example, acrylic acid, methacrylic acid, or a combination thereof. Examples of hydrophilic acrylamide derivatives include, but are not limited to, N,N-diethylacrylamide, 2-[N,N-dimethylamino]ethylacrylamide, 2-[N,N-diethylamino]ethylacrylamide, N,N-diethylmethacrylamide, 2-[N,N-dimethylamino] ethylmethacrylamide, 2-[N,N-diethylamino]ethylmethacrylamide, or combinations thereof. Examples of hydrophilic acrylic esters include, but are not limited to, 2-[N,N-diethylamino] ethylacrylate, 2-[N,N-dimethylamino]ethylacrylate, 2-[N,N-diethylamino]ethylmethacrylate, 2-[N,N-dimethylamino]ethylmethacrylate, or combinations thereof.

As used herein, a "derivatised carboxylic acid co-monomer" refers to a hydrophilic acryl- or methacryl- carboxylic acid, for example, acrylic acid, methacrylic acid, or a combination thereof, which has been chemically derivatized to contain one or more cross-linking moieties, such as succinimidyl groups. The term "succinimidyl group" is intended to encompass variations of the generic succinimidyl group, such as sulphosuccinimidyl groups. In the context of the present

invention a succinimidyl group acts to increase the reactivity of the carboxylic acid group to which it is attached towards primary amines (*i.e.* $-NH_2$ groups) and thiols (*i.e.* –SH groups). Examples of suitable derivatised carboxylic acid co-monomers for use in the synthetic polymer include, but are not limited to, N-acryloxysuccinimide.

One skilled in the art will appreciate that the selection and ratio of the components in the synthetic polymer will be dependent to varying degrees on the final application of the bio-synthetic matrix. For example, for ophthalmic applications, it is important that the final matrix be clear, whereas for other tissue engineering applications, the clarity of the matrix may not be an important factor. Furthermore, it will be appreciated that if bioactive agents are to be covalently attached (or "grafted") to the polymer, or if the synthetic and biopolymers are to be cross-linked, then a synthetic polymer comprising a derivatised carboxylic acid co-monomer will be useful.

In one embodiment of the present invention, the synthetic polymer is a co-polymer comprising one acrylamide derivative and one hydrophilic co-monomer. In a related embodiment, the synthetic polymer is a co-polymer comprising NiPAAm monomer and acrylic acid (AAc) monomer.

In another embodiment of the present invention, the synthetic polymer is a terpolymer comprising one acrylamide derivative, one hydrophilic co-monomer and one derivatised carboxylic acid co-monomer. In a related embodiment, the synthetic polymer is a terpolymer comprising NiPAAm monomer, acrylic acid (AAc) monomer and a derivatised acrylic acid monomer. In another embodiment, the synthetic polymer is a terpolymer comprising NiPAAm monomers, acrylamide (AAm) monomers and a derivatised acrylic acid monomer. In a related embodiment, the derivatised acrylic acid monomer is N-acryloxysuccinimide. In another related embodiment of the present invention, the terpolymer comprises NiPAAm monomer, AAc monomer and N-acryloxysuccinimide in a ratio of about 85:10:5 mol %.

1.2 Bio-polymer

Bio-polymers are naturally-occurring polymers, such as proteins and carbohydrates. In accordance with the present invention, the bio-synthetic matrix comprises a bio-polymer. The

bio-polymer may be simply mixed with the synthetic polymer, or it may be cross-linked to the synthetic polymer. Methods of cross-linking polymers are well-known in the art and include, for example, the use of cross-linking agents such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimde. Alternatively, the synthetic and bio-polymers can be cross-linked by means of pendant cross-linking moieties in the latter, in which case the bio-polymer contains one or more groups which are capable of reacting with the cross-linking moiety (*e.g.* a primary amine or a thiol).

Examples of suitable bio-polymers for use in the present invention include, but are not limited to, collagens, denatured collagens (or gelatins), fibrin-fibrinogen, elastin, glycoproteins, alginate and glucosaminoglycans. One skilled in the art will appreciate that some of these bio-polymers may need to be derivatised if they are to be cross-linked with the synthetic polymer, for example, glucosaminoglycans can be deacetylated or desulphated in order to possess a primary amine group. Such derivatisation can be achieved by standard techniques and is considered to be within the ordinary skills of a worker in the art.

2. *Preparation of the Bio-Synthetic Matrix*

In accordance with the present invention, the bio-synthetic matrix is a hydrogel comprising a synthetic polymer and bio-polymer. The synthetic polymer can be prepared using standard methods known in the art to co-polymerise of the monomers selected as components for the synthetic polymer. Typically appropriate quantities of the selected monomers are dispersed in a suitable solvent in the presence of an initiator. The mixture is maintained at an appropriate temperature and the co-polymerisation reaction is allowed to proceed for a pre-determined period of time. The resulting polymer can then be purified from the mixture by conventional methods, for example, by precipitation.

The solvent for the co-polymerisation reaction may be an aqueous solvent or it may be a non-aqueous solvent. Suitable aqueous solvents include, but are not limited to, water, buffers and salt solutions. Suitable non-aqueous solvents are typically hydrocarbons, such as dioxane, a chlorinated hydrocarbon (for example, chloroform) or an aromatic hydrocarbon (for example,

benzene). In one embodiment of the present invention, the solvent is a non-aqueous solvent. In a related embodiment, the solvent is dioxane.

Suitable polymerisation initiators are known in the art and are usually free-radical initiators. Examples of suitable initiators include, but are not limited to, 2,2'-azobisisobutyronitrile (AIBN).

The hydrogel can be readily prepared by mixing appropriate quantities of the synthetic and bio-polymers and allowing the mixture to gel at a suitable temperature. Alternatively, the mixture can be allowed to dry and can subsequently be re-hydrated prior to use. Additional components may be required to facilitate hydrogel formation as are known in the art. For example, when using fibrinogen as the bio-polymer, the addition of thrombin to the mixture facilitates the formation of fibrin gels.

If cross-linking between the synthetic and bio-polymers is desired, this can also be readily achieved using standard techniques. Methods of cross-linking polymers are well-known in the art and include, for example, the use of cross-linking agents such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide. Alternatively, for synthetic polymers that contain pendant cross-linking groups, cross-linking can be achieved by mixing appropriate amounts of synthetic and bio-polymer at room temperature in an appropriate solvent. Typically the solvent is an aqueous solvent, such as a salt solution, buffer solution, cell culture medium, or a diluted or modified version thereof. The significant levels of amino acids in nutrient media normally used for cell culture can cause side reactions with succinimidyl groups and other cross-linking moieties, which can result in diversion of these groups from the cross-linking reaction. Use of a medium free of amino acids and other proteinacious materials can help to prevent these side reactions and, therefore, increase the number of cross-links that form between the synthetic and bio-polymers. Conducting the cross-linking reaction in aqueous solution at room or physiological temperatures allows both cross-linking and the much slower hydrolysis of any unreacted succinimidyl groups to take place. If necessary, after the cross-linking step, the temperature of the cross-linked polymer suspension can be raised to allow the hydrogel to form.

One skilled in the art will understand that the amount of each polymer to be included in the hydrogel will be dependent on the choice of polymers and the intended application for the hydrogel. In general using higher initial amounts of each polymer will result in the formation of a more robust gel due to the lower water content. The presence of cross-links will also strengthen the hydrogel and alter its elasticity. In one embodiment of the present invention, the final hydrogel contains about 95 % by weight of water.

The relative amounts of each polymer to be included in the hydrogel similarly will be dependent on the type of synthetic polymer and bio-polymer being used and upon the intended application for the hydrogel. In one embodiment of the present invention the ratio of synthetic polymer : bio-polymer is between 1 : 1 and 1 : 3.

3. Incorporation of Bioactive Agents into the Bio-synthetic Matrix

Bioactive agents can be optionally incorporated into the matrix either by covalent attachment (or "grafting") to the synthetic polymer through the pendant cross-linking groups, or by encapsulation within the matrix.

When the bioactive agent is grafted onto the polymer, it can either be attached through a pendant cross-linking group on the synthetic polymer or it can be cross-linked to the synthetic or bio-polymer by means of a cross-linking agent such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide. For covalent attachment of a bioactive agent, the synthetic polymer is first reacted with the bioactive agent and a cross-linking agent of required and then subsequently cross-linked to the bio-polymer as described above.

Bioactive agents which are not suitable for grafting to the polymer can be entrapped in the final matrix. For entrapment, the bioactive agent is added to a solution of the synthetic polymer in an appropriate solvent prior to mixture of the synthetic polymer and the bio-polymer to form a cross-linked hydrogel. Alternatively, the bioactive agent can be added to a solution containing both the synthetic and bio-polymers prior to the cross-linking step. The bioactive agent is mixed into the polymer solution such that it is substantially uniformly dispersed therein, and the hydrogel is subsequently formed as described above. Appropriate solvents for use with the

bioactive agent will be dependent on the properties of the agent and can be readily determined by one skilled in the art.

4. Entrapment of Cells in the Bio-synthetic Matrix

The bio-synthetic matrix according to the present invention may also comprise cells entrapped therein to permit outgrowth of the cells to form an artificial tissue *in vitro*. A variety of different cell types may be incorporated into the bio-synthetic matrix, for example, myocytes, adipocytes, fibromyoblasts, ectodermal cells, muscle cells, osteoblasts (*i.e.* bone cells), chondrocytes (*i.e.* cartilage cells), endothelial cells, fibroblasts, pancreatic cells, hepatocytes, bile duct cells, bone marrow cells, neural cells, genitourinary cells (including nephritic cells), or combinations thereof.

Cells can be readily entrapped in the final matrix by addition of the cells to a solution of the synthetic polymer prior to admixture with the bio-polymer to form a cross-linked hydrogel. Alternatively, the cells can be added to a solution containing both the synthetic and bio-polymers prior to the cross-linking step. The synthetic polymer may be reacted with a bioactive agent prior to admixture with the cells if desired. Typically, for the encapsulation of cells in the matrix, the various components (cells, synthetic polymer and bio-polymer) are dispersed in an aqueous medium, such as a cell culture medium or a diluted or modified version thereof. The cell suspension is mixed gently into the polymer solution until the cells are substantially uniformly dispersed in the solution, then the hydrogel is formed as described above.

5. Preparation of Artificial Tissue

In accordance with the present invention, artificial tissue is constructed on a suitable bio-matrix scaffold. Growth of cells over and / or into the bio-synthetic matrix scaffold can be readily achieved *in vitro* using standard cell culture techniques. For example, cells from one or more appropriate cell lines, such as human endothelial or epithelial cells, can be seeded either directly onto the matrix or onto an appropriate material surrounding the matrix. After growth in the presence of a suitable culture medium for an appropriate length of time, histological examination of the matrix can be conducted to determine whether the cells have grown over the surface of

and/or into the matrix. Alternatively, if cells have been encapsulated in the bio-matrix, the matrix can be cultured in an appropriate medium and out-growth of the cells can be assessed after a suitable time. The present invention contemplates a variety of cell lines for this purpose. Typically cell lines with extended lifespans, such as immortalised cell lines, are used. The use of vascular cell lines, such as human vascular endothelial cells, can allow the development of blood vessel-like structures in or on the artificial tissue. One skilled in the art will appreciate that the cell line will be selected depending upon what type of tissue is being emulated.

6. Innervation of Artificial Tissue or Tissue Substitute

The artificial tissue according to the present invention is innervated. Innervation of the bio-matrix based artificial tissue as described above, or other artificial tissues or tissue substitute is achieved by culturing the "tissue" under appropriate conditions in the presence of a nerve source. An example of a suitable nerve source is dorsal root ganglia, which can be embedded into the artificial tissue or into an appropriate material surrounding the tissue. The artificial tissue is incubated in the presence of a suitable culture medium for an appropriate length of time to permit neural growth. The culture medium and or the bio-synthetic matrix may contain additional substances known in the art to promote nerve growth, for example, additional nutrients, growth factors, differentiating factors and the like. In one embodiment of the present invention, nerve growth factor, and retinal acetate are used to promote nerve growth. Protease inhibitor may also be used to prevent degradation of the bio-synthetic matrix. Examination of the tissue, directly and / or in the presence of a nerve-specific marker, for example by immunofluorescence using a nerve-specific fluorescent marker, will indicate the extent of neural in-growth.

APPLICATIONS

The innervated artificial tissues according to the present invention can be used as *in vitro* alternatives to animals in the toxicological and irritancy testing of a variety of products including, but not limited to pharmaceuticals, diagnostics, household products, cosmetics, personal care products and industrial products.

The artificial tissues can also be used as research tools for investigation of the role of nerves in the various processes, such as wound healing.

In one embodiment of the present invention, the innervated artificial tissue is formed as an artificial cornea. For this application, the tissue is based on a bio-synthetic matrix designed to have a high optical transmission and low light scattering. For example, bio-sythetic matrices comprising a synthetic pNiPAAm-co-AAc co-polymer or a pNiPAAm-co-AAc-co-N-acryloxysuccinimide terpolymer and collagen have high optical transmission, very low light scattering and are capable of remaining clear up to 55°C. The artificial cornea can be prepared by admixture of the synthetic and bio-polymers and injection of the resultant mixture into a suitable mould. If required, the matrix can be cross-linked at room temperature. The incubation temperature can then be raised to about 37°C to allow for the formation of the final hydrogel. For artificial corneas formed from the terpolymer, extensive washing is then performed to remove N-hydroxysuccinimide produced by the cross-linking reaction and to terminate any unreacted cross-linking groups remaining in the matrix prior to use. This artificial cornea is suitable for use in ocular eye irritancy tests as a substitute for current animal models.

3. KITS

The present invention also contemplates kits comprising the components required to prepare an innervated artificial cornea. The kits may comprise a suitable bio-synthetic matrix, cell lines, nerve source, or combinations thereof. The kits may comprise a "ready-made" form of the matrix or they may comprise the individual components required to make the matrix (*i.e.* the synthetic polymer, with or without attached bioactive agents, and the bio-polymer) in appropriate proportions. The kits may further comprise media, appropriate cell culture additives, containers, solvents, or a combination thereof. Individual components of the kit may be packaged in separate containers. The kit may further comprise instructions for use.

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

EXAMPLE 1:PREPARATION OF HYDROGELS

Abbreviations

RTT:	rat-tail tendon
ddH_2O:	distilled, de-ionised water
PBS:	phosphate buffered saline
D-PBS:	Dulbecco's phosphate buffered saline
AIBN:	azobis-isobutyronitrile
NiPAAm:	N-isopropylacrylamide
pNiPAAm:	poly(N-iso-propylacrylamide)
AAc:	acrylic acid
ASI:	N-acryloxysuccinimide
pNIPAAm-co-AAc:	copolymer of NiPAAm and AAc
poly(NiPAAm-co-AAc-co-ASI):	terpolymer of N-isopropylacrylamide, (NIPAAM), acrylic acid (AAc) and N-acryloxysuccinimide (ASI)
GPC:	gel permeation chromatography

All gel matrices described in the Examples set out below used sterile collagen I, such as telocollagen (rat-tail tendon, RTT) or atelocollagen (bovine or porcine), which can be prepared in the laboratory or more conveniently is available commercially (for example, from Becton Dickinson at a concentration of 3.0-3.5 mg/ml in 0.02N acetic acid). Such collagens can be stored for many months at 4°C. In addition, such collagen solutions may be carefully concentrated to give optically clear, very viscous solutions of 3 – 4 wt/vol % collagen, suitable for preparing more robust matrices.

Collagen solutions are adjusted to physiological conditions, *i.e.* saline ionic strength and pH 7.2 – 7.4, through the use of aqueous sodium hydroxide in the presence of phosphate buffered saline

(PBS). PBS, which is free of amino acids and other nutrients, was used to avoid depletion of cross-linking reactivity by side reactions with $-NH_2$ containing molecules.

1.1 Preparation Of A Collagen-pNiPAAm Hydrogel

PNiPAAm homopolymer powder is available commercially (for example, from Polyscience). All other polymers were synthesized as outlined below.A 1 wt/vol% solution of pNiPAAm homopolymer in ddH_2O was sterilised by autoclaving. This solution was mixed with sterile RTT collagen solution [3.0-3.5 mg/ml (w/v) in acetic acid (0.02N in water] (1:1 vol/vol) in a sterile test tube at 4°C by syringe pumping to give complete mixing without bubble formation. Cold mixing avoids any premature gelification or fibrilogenesis of the collagen. The collagen-pNiPAAm was then poured over a plastic dish (untreated culture dish) or a mould (*e.g.* contact lens mould) and left to air-dry under sterile conditions in a laminar flow hood for at least 2-3 days at room temperature. After drying to constant weight (~7 % water residue), the formed matrix was removed from the mould. Removal of the matrix from the mould is facilitated by soaking the mould in a sterile PBS at room temperature. Continued soaking of the free sample in this solution gives a gel at physiological pH and ionic strength, suitable for cell growth.

1.2 Preparation Of A Synthetic Terpolymer

A collagen-reactive terpolymer, poly(NiPAAm-co-AAc-co-ASI) (Figure 1), was synthesised by co-polymerising the three monomers: N-isopropylacrylamide, (NiPAAm), acrylic acid (AAc) and N-acryloxysuccinimide (ASI). The feed molar ratio was 85:10:5 (NiPAAm: AAc: ASI), the free-radical initiator AIBN and the solvent, dioxane, nitrogen purged.

After purification by repeated precipitation to remove traces of homopolymer, the composition of the synthesised terpolymer (82% yield) was found to be 84.2:9.8:6.0 (molar ratio) by proton NMR. The M_n of the terpolymer is 5.6×10^4 Da by aqueous GPC.

A solution of 2 mg/ml of the terpolymer in D-PBS remained clear even up to 55°C. A solution of 10 mg/ml in D-PBS became only slightly cloudy at 43°C. Failure to remove homopolymer formed in the batch polymerisation reaction (due to the NiPAAm reactivity coefficient being

greater than that of AAc or ASI) from the terpolymer gave aqueous solutions and hydrogels which cloud at ~32°C and above.

1.3 Preparation Of A Synthetic Polymer Comprising A Bioactive Agent

A terpolymer, containing the pentapeptide YIGSR (a nerve cell attachment motif), was synthesised by mixing the terpolymer prepared in Section 1.2 (1.0 g) with 2.8μg of laminin pentapeptide (YIGSR, from Novabiochem) in N,N-dimethyl formamide. It was assumed that all the YIGSR pentapeptide was attached to the reactive terpolymer because the pendant, reactive ASI groups are in large excess. ASI groups remaining after reaction with the pentapeptide are available for subsequent reaction with collagen.

1.4 Preparation Of A Collagen-Terpolymer Hydrogel

A cross-linked, terpolymer-collagen hydrogel was made by mixing neutralised 4% bovine collagen (1.2 ml) with the terpolymer prepared in Section 1.2 [0.14ml (100 mg/ml in D-PBS)] by syringe mixing. After careful syringe plumping to produce a homogeneous, bubble-free solution, aliquots were injected into plastic, contact lens moulds and incubated at room temperature for 24 hours to allow reaction of the collagen $-NH_2$ groups with ASI groups as well as the slower hydrolysis of residual ASI groups to AAc groups. The moulded samples were then incubated at 37°C for 24 hours in 100% humidity environment, to give a final hydrogel. The hydrogel contained 95.4 ± 0.1% water, 2.3% collagen and 1.6% terpolymer. Matrices were moulded to have a final thickness between either 150 - 200 μm or 500 – 600 μm. Each resulting hydrogel matrix was removed from its mould under PBS solution and subsequently immersed in PBS containing 1% chloroform and 0.5% glycine. This wash step removed N-hydroxysuccinimide produced in the cross-linking reaction and terminated any unreacted ASI groups in the matrix, by conversion to acrylic acid groups.

Succinimide residues left in the gels prepared from collagen and terpolymer were below the IR detection limit after washing.

1.5 Preparation Of A Hydrogel Comprising A Bioactive Agent

Cross-linked hydrogels of collagen-terpolymer comprising YIGSR cell adhesion factor were prepared by thoroughly mixing viscous, neutralised 4% bovine collagen (1.2 ml) with terpolymer to which laminin pentapeptide (YIGSR) was covalently attached (from Section 1.3; 0.14ml, 100 mg/ml) at 4°C, following the procedure described in Section 1.4.

1.6 Comparison Of The Physical Properties Of Hydrogel Matrices

Collagen thermogels (prepared at 37°C, without any chemical cross-linker added) are frail and readily collapse and break.

The following properties of the hydrogels prepared as described in Examples 4 and 5 indicate that they are cross-linked:

- water insoluble,
- strong enough to support surgical manipulation with suture thread and needle
- relatively flexible in handling
- demonstrate an increase in stress at failure and apparent modulus during tensile testing by over x2 on going from $-NH_2$/ASI equivalent ratio of 0.5 to 1.5.

The hydrogels prepared as described in Section 1.4 and 1.5 have high optical transmission and very low light scattering, comparable to the human cornea, as measured with a custom-built instrument that measures transmission and scatter (Priest and Munger, 1998). In contrast, collagen- pNiPAAm homopolymer gels (as described in Section 1.1; 1.0 : 0.7 to 1.0 : 2.0 wt/wt) were opaque at 37°C. In addition, the pNiPAAm homopolymer in gels from Example 1 extracts out into aqueous media, including physiological liquids.

EXAMPLE 2: FUNCTIONAL INNERVATION OF A TISSUE ENGINEERED HUMAN CORNEA

2.1 Tissue Engineering Innervated Corneas and Nerve Growth Patterns

Cell lines with extended lifespans [M. Griffith *et al.*, *Science* 286, 2169 (1999)] were used to develop tissue engineered (TE) corneas as substrates for nerve innervation. The cell lines included a SV40 immortalized corneal epithelial cell line known to have the appropriate receptors (neurokinin-1, NK1) for the Substance P (SP) neurotransmitter [K. Araki-Sasaki *et al.*, *J. Cell Physiol.* 182, 189 (2000)] and human papilloma virus (HPV) 16 E6E7 immortalized corneal stroma, corneal endothelial and human umbilical vein endothelial cell lines (HUVECs) [M. Griffith, *et al.*, in *Methods in Tissue Engineering*, A. Atala, R. P. Lanza, Eds. (Academic Press, San Diego, CA, 2002), Chap. 9].

Dorsal root ganglia (DRG) dissected from eight day old chick embryos served as the nerve source. DRG were embedded in an annular, collagen-containing hydrogel that served as a scleral scaffold, within the centre of which a cornea was fabricated (Fig. 2A). In more detail, DRG, isolated by collagenase digestion and micro-dissection, were embedded in a ring of neutralised, type I rat tail tendon collagen (0.3% (w/v), Becton-Dickinson) with chondroitin 6-sulfate (1.5% (w/v)) which had been previously cross-linked with 0.02% v/v glutaraldehyde (followed by glycine termination) and thermo-gelled at 37°C for 2 hours. A cornea was fabricated within this collagen ring, using a blend of neutralised type I rat tail tendon collagen and chondroitin-6-sulphate (Sigma). A laminin (Becton-Dickinson) gradient was created within the stroma to promote the growth of nerves towards the epithelium. Three layers were made with concentrations increasing from bottom to top (0, 10 and 20 μg/ml). This formulation was then cross-linked with 0.02% glutaraldehyde. Residual aldehyde groups were reacted with a 0.8% aqueous glycine (w/v) solution (details in M. Griffith, *et al.*, in *Methods in Tissue Engineering*, A. Atala, R. P. Lanza, Eds. (Academic Press, San Diego, CA, 2002), Chap. 9). The construct was then thermo-gelled by incubation at 37°C for 2 hours. The cultures were supplemented with a modified SHEM medium (11) containing 2% B27 and 1% N2 supplements (Life Technologies). Optimized concentrations of 1 nM retinal acetate (RA; Sigma) and 100 ng/ml nerve growth factor (NGF; Sigma) were added to the growth medium and upper corneal layer, respectively, to induce nerve in-growth. At epithelial confluence, the constructs were airlifted and maintained at an air-liquid interface for up to 10 days until used.

Whole mount immunofluorescence with nerve specific anti-neurofilament antibody markers was used to analyse the innervation of the artificial cornea. Cornea constructs were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), and then permeabilized by treatment with RIPA detergent (150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate) for 20 minutes. They were rinsed in Tris buffered saline (TBS), and incubated with anti-neurofilament 200 (Sigma; diluted 1:40 in TBS containing 0.6% carrageenan and 0.3% Triton-X 100 (TCT)) over 2 nights at 4°C. The constructs were then rinsed in TBS and incubated with a Cy3-conjugated secondary antibody (1:200 in TCT; Amersham) for 150 minutes at room temperature (RT). Negative controls were incubated without the primary antibody. Positive controls included staining of DRG and neural tube explants. Nerve growth patterns identical to those observed in human corneas were demonstrated in fabricated corneas. Nerves bundles from the DRG within the scleral scaffold coursed through the corneal stroma (Fig. 2B) and bifurcated with successively finer branches to form a plexus (Fig. 2C) below the basal epithelial cells. As in natural corneas, many bundles of this nerve plexus ran parallel to each other with bifurcations running at near right angles. Both beaded and smooth nerve fibres from the sub-epithelial network proceeded to target and migrate within the epithelium (Fig. 2D). Transmission electron microscopy (TEM) showed terminal nerve fibres invaginated corneal epithelial cells (Fig. 2E, F), as previously described for human corneas [L. J. Müller, L. Pels, G. F. J. M. Vrensen, *Invest. Ophthalmol. Vis. Sci.* 37, 476 (1996)], suggesting that these cells receive direct innervation.

2.2 *Nerve Action Potentials within Tissue Engineered Corneas*

Sodium channels are integral to the generation of nerve action potentials. Action potentials propagate from axons to the central nervous system to cause pain, and also to the nerve terminals within the epithelium to cause the release of neuropeptides.

Immunohistochemistry was conducted on the innervated artificial corneas. Briefly, paraformaldehyde-fixed constructs were rinsed in 0.05 M Tris buffer, pH 7.4 and permeabilized in Tris buffer containing 0.3% Triton X-100. Following blocking with 10% normal goat serum in buffer, tissues were incubated overnight at 4°C with the primary antibody, monoclonal anti-PAN sodium channel antibody (Sigma), at a dilution of 1:250 in Tris buffer containing 2% normal

goat serum. The tissues were then rinsed thoroughly in Tris buffer and reacted with a 1:100 dilution of secondary antibody, goat anti-mouse Alexa 488 (Molecular Probes), in Tris buffer for 90 minutes prior to visualization under fluorescence microscopy. Sodium channels were observed in the nerve fibres of the TE corneas (Fig. 3A, B), indicating that the axons most likely possess the machinery necessary to be excitable and functional.

Direct electrophysiological recording from the corneal epithelium was therefore conducted to confirm that nerve bundles growing into the cornea were able to conduct lidocaine-sensitive action potentials that were evoked by stimulation of the ganglion cell cluster (Fig. 3C, D). Tissue engineered corneas were transferred into an interface recording chamber, perfused with artificial saline containing (in mM): NaCl: 126, KCl: 3.0, $MgSO_4$: 2.0, $NaHCO_3$: 26, NaH_2PO_4 1.25, $CaCl_2$: 2.0, dextrose: 10, oxygenated with 95% O2 / 5% CO2 at room temperature. Cathodal stimulation of ganglion cell clusters was done using silver wires pressed lightly against the surface and applying square wave stimulus pulses of 50 μs duration and typically 60-80 V in amplitude. Differential recordings of electrical responses from nerve fibre bundles were recorded with glass micropipettes (≈ 50 μm tips) filled with 150 mM NaCl. Because of the close proximity of the stimulation to the recording electrodes, a very large stimulus artefact was generated that obscured the very small action potentials (Fig. 3C). To observe action potentials in isolation, evoked responses were recorded before and after addition of 50 mM lidocaine HCl. Subtracting the responses in lidocaine from control responses yielded isolated action potentials with the stimulus artefacts largely removed (Fig. 3D). These action potentials exhibited a configuration and amplitude similar to those recorded from nerve endings in guinea-pig corneas [J. A. Brock, E. M. McLachlan, C. Belmonte, *J. Physiol.* 512, 211 (1998)]. The generation of action potentials is important to the function of the corneal nerve endings in the epithelium.

2.3 Effects of Innervation on Wound Healing and Response to Chemicals

The loss of corneal innervation is known to reduce epithelial cell proliferation and to slow wound healing in rabbit corneas. To test whether this effect was reproducible in the artificial cornea system, epithelial wounds were created in TE corneas constructed with and without nerves, and wound closure rates were measured. To create wounds, a circle of filter paper (3mm diameter) was placed on the epithelium of each construct, allowed to adhere and then peeled off, leaving an

area devoid of epithelial cells, as determined by scanning electron microscopy (SEM) on random samples. Wound closure (re-epithelialization) was determined at 0, 6, 12, 18, 24, 36, 48 and 72 hours post-wounding by microscopy, with area calculated using BioRad Quantity One© software. Mean initial wound areas of the innervated group (6.68 ± 0.17 mm^2) and non-innervated group (7.02 ± 0.11 mm^2) were not significantly different (t-test, P=0.21). To account for variation in original wound sizes, a new healing parameter that is independent of the original wound area was developed. Since wound healing is dependent upon the number of cells at the wound edge that can migrate into the wound or multiply to cover the wound, the number of progenitor cells at the wound edge is proportional to the circumference of the wound. By dividing the change in area of the wound by the original wound circumference, we obtain a measure of the healing that has occurred per number of progenitor cells. This new normalised healing parameter is now independent of the original wound area.

During the first 18 hours, the innervated corneal constructs showed a higher rate of wound closure (Fig. 4A). By 24 hours, however, no differences in total wound healing were observed between the innervated constructs and non-innervated controls. Bromodeoxyuridine (BrdU, a mitotic indicator) incorporation at 0, 6 and 24 hours post-wounding showed an increase in the percentage of labeled epithelial cells in innervated constructs compared to non-innervated controls (Fig. 4B). These results demonstrate that the presence of nerves in the TE cornea promotes proliferation of epithelial cells, and are consistent with previous *in vivo* rabbit studies [J. Garcia-Hirschfeld, L. G. Lopez-Briones, C. Belmonte, *Exp. Eye Res.* 59, 597 (1994)]. However, no significant changes ($p > 0.05$) in the mitotic index were observed within either group over the first 24 hours after wounding. This suggests that the higher rate of wound closure of innervated constructs over the initial 18 hours (Fig. 4A) is most likely due to faster epithelial cell migration. This observation is supported by previous reports that the presence of nerves promotes migration of corneal epithelial cells [R. W. Beuerman, B. Schimmelpfennig, *Exp. Neurol.* 69, 196 (1980)]. These data are also consistent with *in vivo* rabbit studies that demonstrate increased epithelial cell proliferation begins in the wound area only 24 hours after wounding [L. Gan, H. Hamberg-Nystrom, P. Fagerholm, G. Van Setten, *Acta. Ophthalmol. Scand.* 79, 488 (2001)].

During cornea wound healing, neuropeptides such as SP are released from nerve terminals and are believed to promote healing effects associated with corneal innervation [T. Nishida *et al.*, *J. Cell Physiol.* 169, 159 (1996); M. Nakamura, *et al.*, *Curr. Eye Res.* 16, 275 (1997)]. Furthermore, the absence of neuropeptides in corneal nerves has been correlated with delayed corneal wound healing [J. Gallar, *et al.*, *Invest. Ophthalmol. Vis. Sci.* 31, 1968 (1990)]. SP has been shown to exert a stimulatory effect on corneal epithelial cell proliferation and migration [J. Garcia-Hirschfeld, *et al.*, *Exp. Eye Res.* 59, 597 (1994); T. Nishida *et al.*, *J. Cell Physiol.* 169, 159 (1996)] via the NK1 receptor [M. Nakamura *et al.*, *Br. J. Pharmacol.*, 120, 547 (1997)] and to play a role in epithelial cell adhesion.

To elicit a functional response such as SP release, innervated TE cornea constructs were treated with capsaicin or veratridine. Briefly, innervated tissue engineered corneas were treated with a total of 1.5 ml of SHEM containing 8.5% Tween 80 and 1.5% ethanol either alone (control) or with 1) 1% (w/v) capsaicin, or 2) 50 μM veratridine. At 0, 6 and 24 hours post-treatment, culture supernatants were collected, flash frozen in liquid nitrogen and stored at -80°C. Substance P content of the medium was measured using a substance P specific competitive peptide enzyme immunoassay (EIA) kit (Peninsula Laboratories). A significant increase in SP release from nerve axons in capsaicin-treated samples was observed over 24 hours, compared to capsaicin-free controls (Fig. 4C). Differential SP release was seen when levels of the neuropeptide were compared amongst innervated corneal constructs treated with capsaicin, veratridine or drug vehicle only (Fig. 4D). At 6 hours post-treatment, only capsaicin elicited a significant increase in SP release, whereas at 24 hours, both capsaicin and veratridine elicited significant increases. Capsaicin is a neurotoxin that depletes SP from peripheral nerve terminal stores by an action potential-independent mechanism that is not fully understood. Veratridine, on the other hand, causes SP release from nerve terminals by opening sodium channels and depolarizing the membrane [J. K. Neubert *et al.*, *Brain Res.* 871, 181 (2000)]. Both sodium channel-dependent and independent mechanisms of SP release were observed in the innervated cornea model. Nerves growing into the TE cornea were therefore capable of both responding to chemical stimuli and conducting action potentials in a fashion similar to native nerve processes.

The presence of nerves in the TE cornea was able to protect the epithelium from chemical irritation. Innervated and non-innervated constructs were exposed to a mixture of 8.5% Tween-80 surfactant and 1.5% ethanol in SHEM medium, and live/dead cell counts were performed (Fig. 5A, B). Sixty-two percent of sampled cells were dead in constructs lacking innervation, compared to innervated constructs in which only 11 % of cells were dead ($n = 3$ each; $p < 0.05$, t-test). This was consistent with the demonstrated role for nerves in the homeostasis of corneal epithelial cells in the human cornea.

EXAMPLE 3: INNERVATION OF ARTIFICIAL TISSUES BASED ON BIO-SYNTHETIC MATRICES

3.1 Fibrin-Polyacrylamide Matrix

A fully innervated cornea surrounded by a pseudo-sclera was prepared using a bio-synthetic matrix as described below. To encourage both innervation and angiogenesis, the pseudo-sclera was constructed by adding HUVECs and DRGs into a blended fibrin-polyamide-laminin scaffold. Like the natural cornea and sclera, the cornea was avascular, while the surrounding sclera contained both nerves and blood vessel-like structures (Fig. 5C).

3.1.1 Co-polymer Synthesis

The co-polymer, poly(N-isopropylacrylamide-co-acrylic acid) [poly(NiPAAm-co-AAc)], was prepared by conventional free-radical polymerisation of NiPAAm 10.75 g (95 mmol) and acrylic acid 0.36 g (5 mmol) in benzene with azobisisobutyronitrile (AIBN) as the initiator. The reaction can also be conducted in 1,4-dioxane. The product (78% yield) was characterized by GPC (molecular weights: $M_n = 41\,039$; $M_w = 70\,968$; GPC was run in distilled water at 30°C, calibrated with polyethylene glycol standards). ^{1}H-NMR was used to determine the monomer ratios after the polymer's acrylic groups had been methylated by BF_3-MeOH reagent. This gave a composition of 95.3 mole % NiPAAm and 4.7 mole % AAc after purification by repeated precipitation to remove traces of homopolymer. Very similar values for the purified composition were obtained by back titration. The NiPAAm -co-AAc at 2 mg/ml has a lower critical solution temperature (LCST) of 54°C in PBS and 41°C in ddH_2O. Failure to remove homopolymer

formed in the batch polymerization reaction (because of the NiPAAm reactivity coefficient being greater than that of AAc) gives aqueous solutions of the product which cloud at ~32°C and above.

A solution of pNiPAAm-co-AAc in ddH_2O can be sterilized by autoclaving or filtering and this solution is stable to storage at room temperature for many months.

3.1.2 Cells and immortalization

Human umbilical vein endothelial cells (HUVECs) were plated on gelatin-coated tissue-culture dishes in medium 199 supplemented with 10% fetal bovine serum (FBS), 90 mg/l of heparin, 2mM of L-glutamine and 50 mg/ml endothelial cells growth supplements (ECGS), bFGF (50 ng/ml) and EGF (10 ng/ml) and 10-12 drops of 10 mg/ml of gentamycin (HUVEC medium).

Primary HUVECs were immortalized through viral infection with Human papilloma virus HP16 E6 E7. After 48 hours, the viral supernatants were removed and the medium replaced with the HUVEC medium.After splitting the cells, selection medium (HUVEC medium with 400 µg/ml antibiotic - G418) was added. Cultures were maintained in selective media for 7 days. The G418-selected cells were then grown in HUVEC medium and further expanded.

Human telomerase reverse transcriptase (hTERT) were used to verify the telomerase activity in the immortalized cells.Endothelial cell phenotype was verified by the di-acetylated low density lipoprotein (di-Ac-LDL) uptake and positivity to factor VIII-related antigen antibody by immunocytochemistry.

3.1.3 Fibrin Matrix

Fibrinogen solution (3 mg/ml) was prepared by dissolving fibrinogen in Hank's balanced salt solution (HBSS) with Ca^{++} and Mg^{++}. The resulted solution was then sterilized by filtering through a 0.22 µm syringe filter. Thrombin solutions were made by dissolving thrombin in HBSS at a concentration of 1.75 mg/ml.

The fibrinogen solution (3mg/ml) was mixed with the thrombin solution (1.75 mg/ml) at a ratio of 1:0.03 v/v in wells of different sizes. Within a minute, enzymatic polymerization of fibrinogen gave fibrin gels under gentle agitation at 37 °C. To incorporate endothelial cells in the fibrin matrix and to induce angiogenesis, endothelial cells were firstly seeded on the bottom of gelatin-coated wells at high density to provide a confluent monolayer at 48 hours. Then, 5×10^4 endothelial cells/ml were dispersed in fibrinogen solution prior to polymerization. Fibrin gels were obtained again within a minute.

3.1.4 Fibrin + P(NiPAAm)-Co-AAc Matrix

Fibrinogen (3mg/ml) was dissolved in Hank's balanced salt solution (HBSS) with Ca^{++} and Mg^{++} and combined with 0.5 % poly(NiPAAm-co-AAc) (NiPAAm:AAC = 95:5) in HBSS at a ratio of 1:1, in the presence of thrombin (1.75 mg/ml in HBSS) at a ratio of 1:0.03 v/v to allow polymerization. To incorporate endothelial cells in the matrix and to induce angiogenesis, endothelial cells were first seeded on the bottom of gelatin-coated wells at high density so as to provide a confluent monolayer after 48 hours. Then, 5×10^4 endothelial cells/ml were dispersed in the solution prior to polymerization as described above (see Fig. 6).

3.1.5 Innervated Cornea And Sclera Model

Dorsal root ganglia were dissected from 8 day old chicken embryos and embedded three-dimensionally inside the Fibrin + P (NiPAAm)-co-AAc gels with 10 µl/ml of laminin and 10 µl/ml of nerve growth factor (NGF). The construct was supplemented with a modified SHEM medium containing 2% B27 and 1% N2 supplements (see Fig. 7).

3.1.6 Detection of Metalloproteinases

Zymography has the advantage that it in addition to detecting enzyme activity it can be used to provide information about the molecular weight of an enzyme and so help identify the enzyme. To achieve this dissolved gelatin is incorporated into a polyacrylamide gel, samples are added to the gel, separated by electrophoresis and the gel allowed to incubate for a while to allow the

enzymes to degrade the gelatin. When the gels are stained for proteins clear lysis bands are apparent where the metalloproteinases have degraded the gelatin (see Fig. 8).

3.1.7 Conclusions

Expression of telomerase activity in immortalized HUVECs was demonstrated. Telomerase activity was not detected in the primary HUVECs indicating that it was possible to immortalize HUVECs to obtain large numbers for use as a cell source for in vitro studies.

Immortalized HUVECs expressed Factor VIII related antigen and took up Dil-Ac-LDL as markers of endothelial origin. The immortalized HUVECs line ressembled normal HUVECs lines, except that they failed to senesce. The preservation of a normal phenotype in immortalized HUVEC allows use of these cells in tissue engineering to realistically mimic native tissues.

Fibrin, PNIPAAM and P(NIPAAM)-co-AAC were used to fabricate hydrated matrices for the three-dimensional culture of HUVECs. The hydrogels were able to interact biologically with cells, inducing proliferation, migration HUVECs ae, therefore, able to form blood vessels within the matrices indicating that the polymers have no toxicity towards the cells, which is important for cell culture applications. Innervate of the three-dimensional sclera was also demonstrated.

The metalloproteases, MMP-2 and MMP-9, were detected in the collagen matrix where neutrophils were added on top of matrix, indicating that FMLP was able to stimulate them.

3.2 Collagen-Polyacrylamide Matrix

A collagen-poly (N-isopropyl polyacrylamide) composite was prepared by blending 1% aqueous poly-N-isopropylacrylamide with 0.3% type I rat tail collagen in 0.02N acetic acid in a 1:1 ratio (v/v). This combined solution was dried down at 20°C to give a thermogel, which was then rehydrated in PBS to give approximately 150 – 200 μm thick hydrogel composites (10% (w/v) total polymers). Use of this matrix *in vitro* as described above resulted in neurite growth into the polymer scaffold was observed *in vitro* (Fig. 5D).

EXAMPLE 4: CONSTRUCTION OF TISSUE ENGINEERED HUMAN CORNEAS FOR IN VITRO TESTING

Cell lines with extended lifespans (7) used to develop TE corneas as substrates for nerve innervation included a SV40 immortalized corneal epithelial cell line known to have the appropriate receptors (neurokinin-1, NK-1) for the Substance P (SP) neurotransmitter (8), and human papilloma virus (HPV) 16 E6E7 immortalized corneal stroma and in a some constructs, endothelial cells (9). DRG, isolated by collagenase digestion and micro-dissection, were embedded in a ring of neutralized, type I rat tail tendon collagen (0.3% (w/v, Becton-Dickinson) which had been previously cross-linked with 0.02% v/v glutaraldehyde (followed by glycine termination) and then thermo-gelled at 37°C for 2 hours.gel that served as a pseudo-sclera. A cornea was then fabricated within this scleral collagen ring, using a blend of neutralized type I rat tail tendon collagen and chondroitin-6-sulphate (1.5% w/v, Sigma).that was A laminin (Becton-Dickinson) gradient was created within the stroma to promote the growth of nerves towards the epithelium. Three layers were made with concentrations increasing from bottom to top (0, 10 and 20 µg/ml). This formulation was then cross-linked with 0.02% glutaraldehyde. Residual aldehyde groups were reacted with a 0.3% aqueous glycine (w/v) solution. The construct was then thermo-gelled by incubation at 37°C for 2 hours. The cultures were supplemented with a modified SHEM medium (11) containing 2% B27 and 1% N2 supplements (Life Technologies). Optimized concentrations of 1 nM retinal acetate (RA; Sigma) and 100 ng/ml nerve growth factor (NGF; Sigma) were added to the growth medium and upper corneal layer, respectively, to induce nerve in-growth. After epithelial confluence, the constructs were airlifted and maintained at an air-liquid interface for up to 10 days until used.

A laminin (Becton-Dickinson) gradient was created within the stroma to promote the growth of nerves towards the epithelium. Three layers were made with concentrations increasing from bottom to top (0, 10 and 20 µg/ml).

When polymers are tested for biocompatibility and their potential for host integration, they are "implanted" into this fabricated cornea by embedding during the construction process. All except the outer convex surface of the hydrogel is embedded within the stroma. The outer surface is exposed to test for epithelial cell coverage.

Results showed that the current collagen-terpolymer-laminin pentapeptide samples were able to support stratified epithelial coverage, and stromal cell and nerve in-growth in culture over a 2-3 week culture period. Collagen-terpolymer alone at some concentrations only allowed sparse epithelial coverage, and in some samples no cellular in-growth into the hydrogel.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

TABLE 1: Blood Vessel Formation in Different Matrices

	Presence of tubes	Longevity (occurrence of fibrinolysis)
Fibrin+0.5% P(NIPAAm)-co-AAC(1:0.1)	++	>10
Fibrin+0.5% P(NIPAAm)-co-AAC(1:1)	+++	>10
Fibrin+0.5% P(NIPAAm)-co-AAC(1:2)	+	<7
Fibrin+0.5% P(NIPAAm)-co-AAC(1:3)	-	<3
Fibrin+1% P(NIPAAm)-co-AAC(1:0.1)	++	>10
Fibrin+1% P(NIPAAm)-co-AAC(1:1)	+	>10
Fibrin+1% P(NIPAAm)-co-AAC(1:2)	+	<7
Fibrin+1% P(NIPAAm)-co-AAC(1:3)	-	<3
Fibrin+2% P(NIPAAm)-co-AAC(1:0.1)	+	<7
Fibrin+2% P(NIPAAm)-co-AAC(1:1)	+	<7
Fibrin+2% P(NIPAAm)-co-AAC(1:2)	+	<7
Fibrin+2% P(NIPAAm)-co-AAC(1:3)	-	<3
Fibrin+3% P(NIPAAm)-co-AAC(1:0.1)	+	<7
Fibrin+3% P(NIPAAm)-co-AAC(1:1)	+	<7
Fibrin+3% P(NIPAAm)-co-AAC(1:2)	+	<7
Fibrin+3% P(NIPAAm)-co-AAC(1:3)	-	<3

Legend:

++ : Many blood vessels

+ : Few blood vessels

- : No blood vessels

TABLE 2: Blood Vessel Formation in Different Matrices

	Presence of tubes	Longevity (occurrence of fibrinolysis)
Fibrin	++	>10
Fibrin+0.5% P(NIPAAm)(1:0.1)		>10
Fibrin+0.5% P(NIPAAm)(1:0.2)	++	>10
Fibrin+0.5% P(NIPAAm)(1:0.3)	+	>10

Legend:
++ : Many blood vessels + : Few blood vessels

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of *in vitro* toxicology testing using an innervated artificial cornea comprising the steps of constructing said cornea from:

   (a) a bio-synthetic matrix comprising a synthetic polymer and a biopolymer;
   (b) one or more cell lines; and
   (c) a nerve source; and

   and contacting said cornea with a test substance.

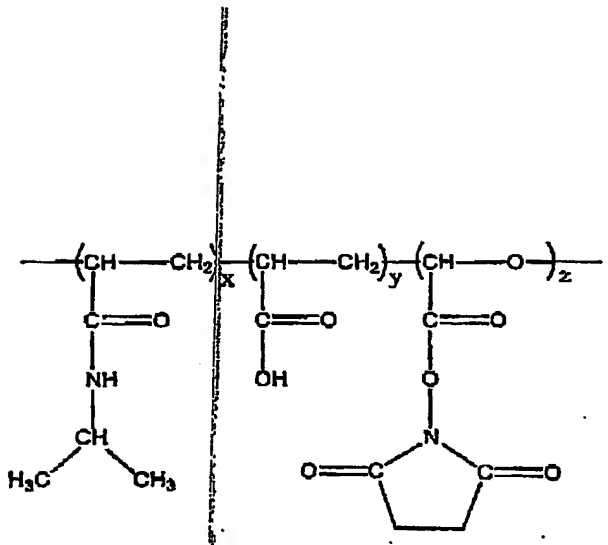


Figure 1

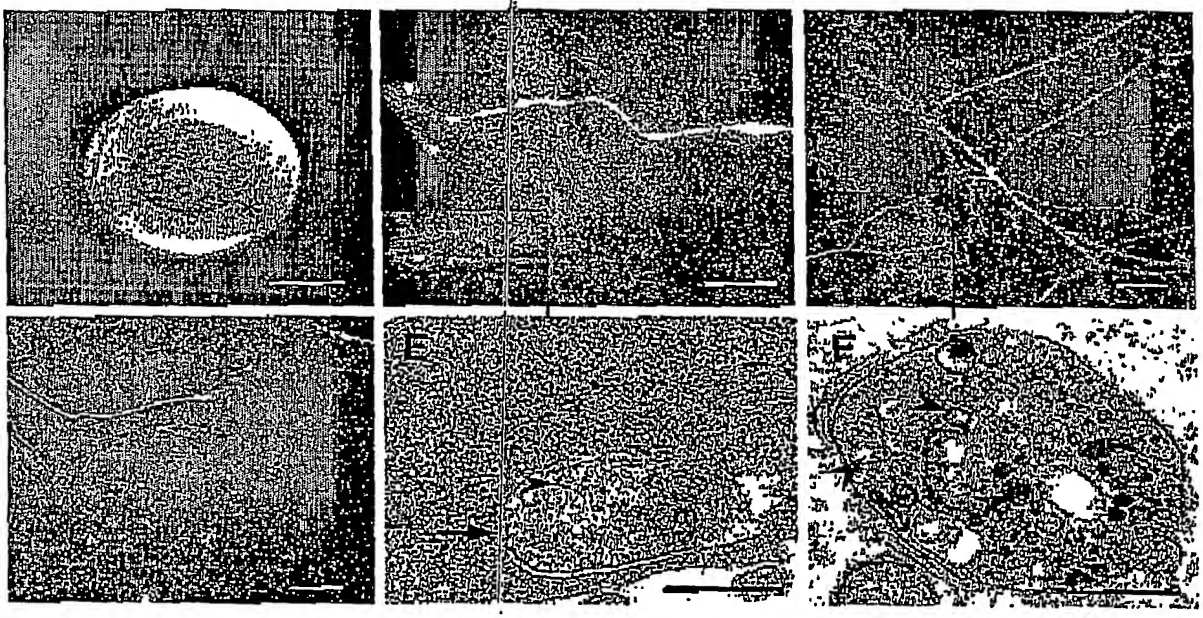
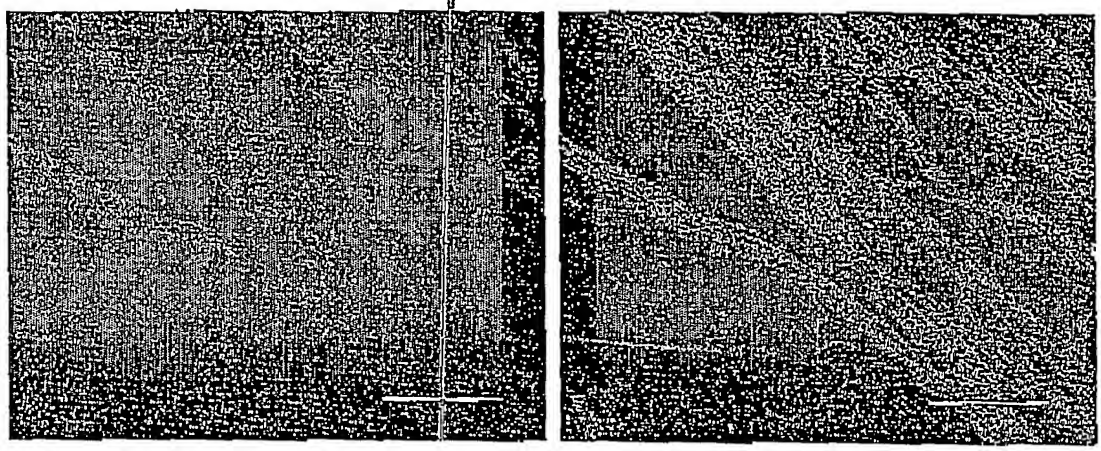


Figure 2



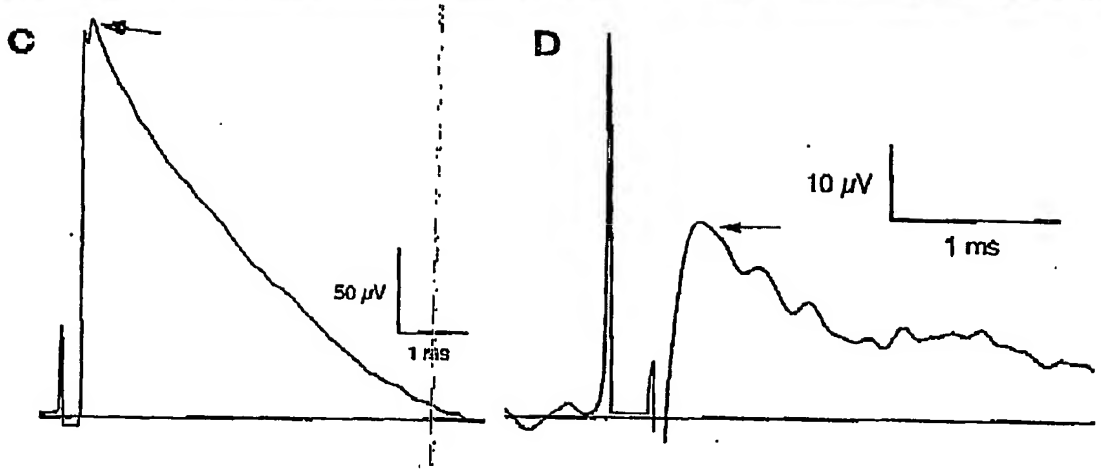


Figure 3

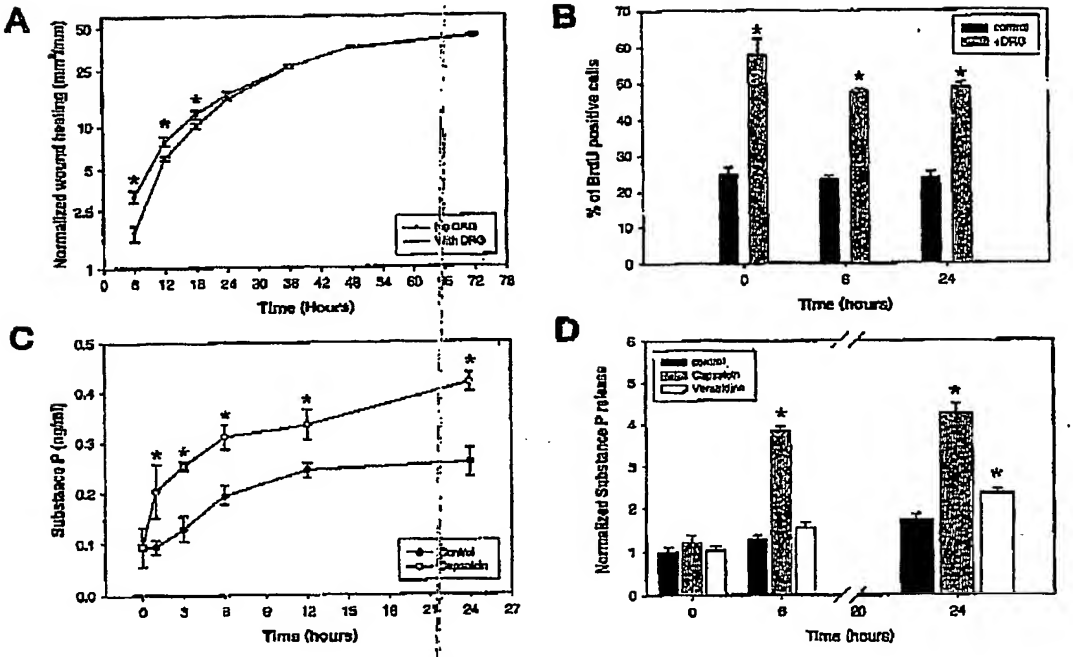

A
Normalized wound healing (mm²/mm)
Time (Hours)
B
% of BrdU positive cells
Time (hours)
C
Substance P (ng/ml)
Time (hours)
D
Normalized Substance P release
Time (hours)

Figure 4

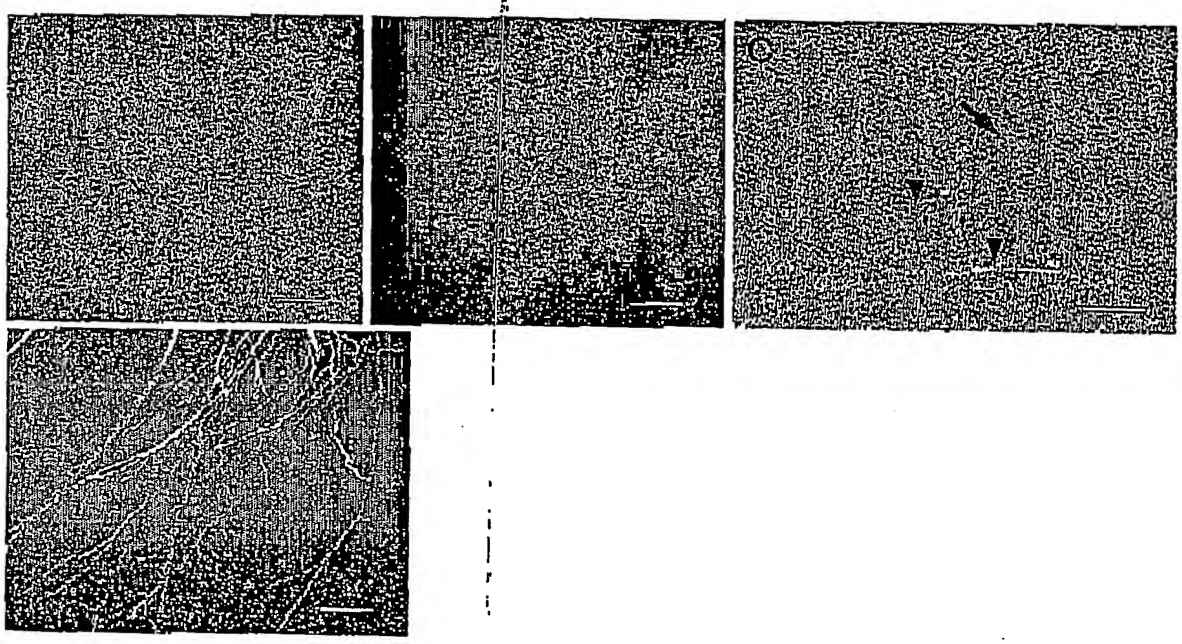


Figure 5

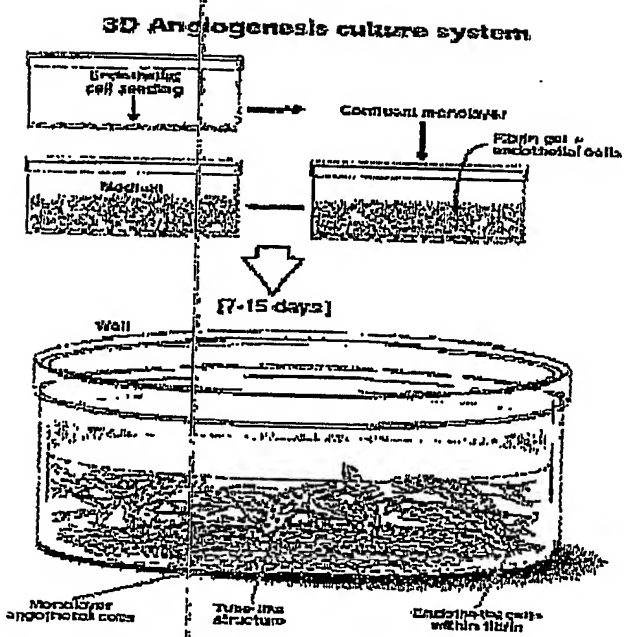

3D Angiogenesis culture system
Endothelial cell seeding
Confluent monolayer
Fibrin gel + endothelial cells
Medium
[7-15 days]
Wall
Monolayer angiothelial cords
Tube-like structure
Endothelial cells within fibrin

Figure 6

Innervated Cornea and Sclera model

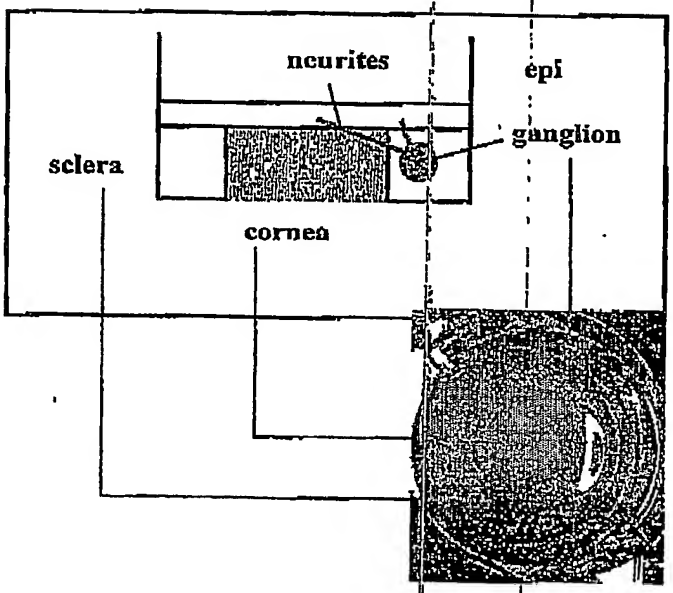
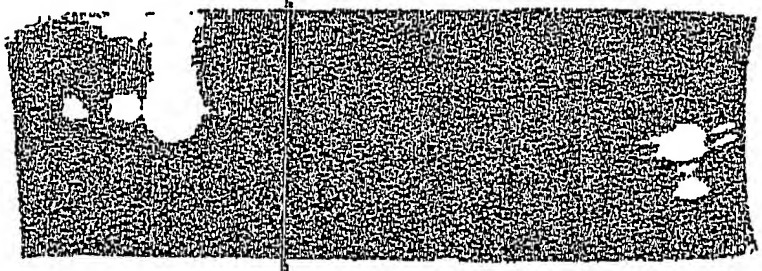


Figure 7

Detection of Metalloproteinases



Samples :

Well	Sample
1	-
2	MMP-9 (2 ng)
3	MMP-9 (10 ng)
4	Activated MMP-9 (2 ng)
5	Neutrophils (Collagen) FMLP 10^{-7} T = 4 h
6	Neutrophils (Collagen) FMLP 10^{-7} T = 6 h
7	Neutrophils (Collagen) FMLP 10^{-7} T = 8 h
8	Neutrophils (Collagen) FMLP 10^{-6} T = 4 h
9	Neutrophils (Collagen) FMLP 10^{-6} T = 6 h
10	Neutrophils (Collagen) FMLP 10^{-6} T = 8 h
11	Neutrophils (Collagen) FMLP 10^{-5} T = 4 h
12	Neutrophils (Collagen) FMLP 10^{-5} T = 6 h
13	Neutrophils (Collagen) FMLP 10^{-5} T = 8 h
14	MMP-2 (10 ng)
15	MMP-2 (2 ng)

Figure 8

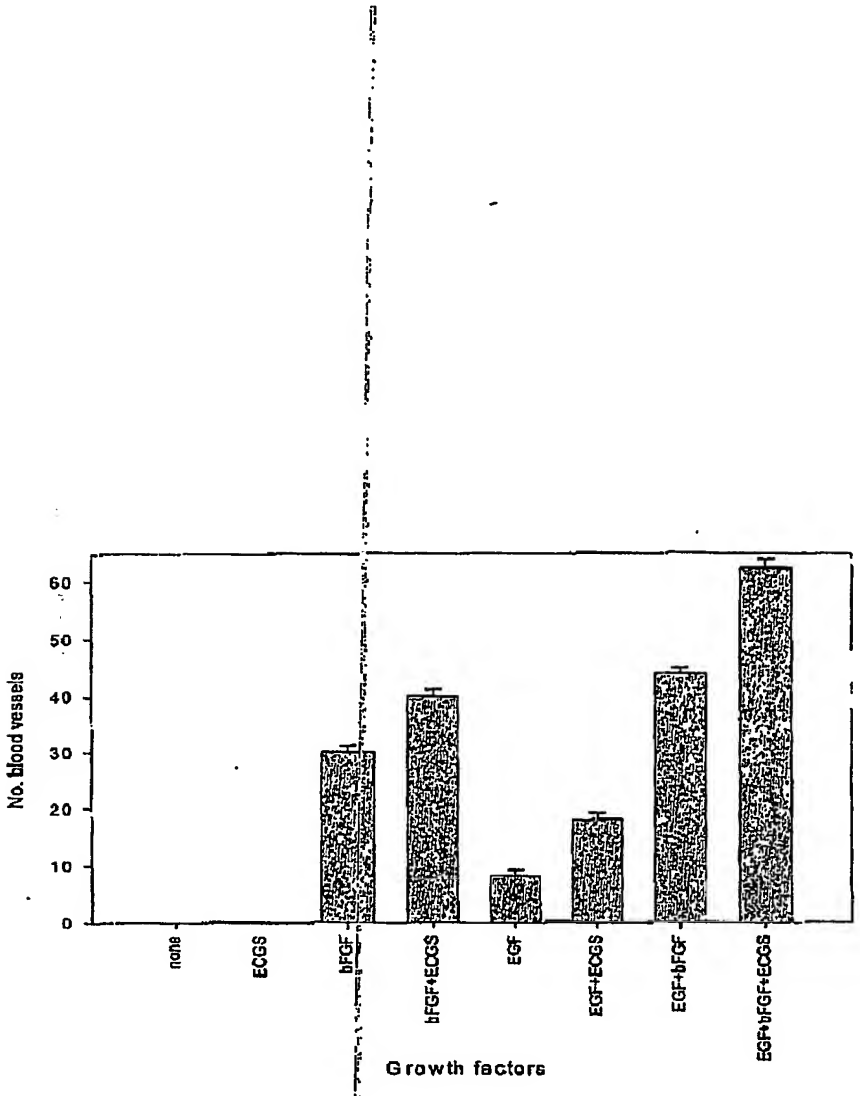

No. blood vessels
0
10
20
30
40
50
60
none
ECGS
bFGF
bFGF+ECGS
EGF
EGF+ECGS
EGF+bFGF
EGF+bFGF+ECGS
Growth factors

Figure 9

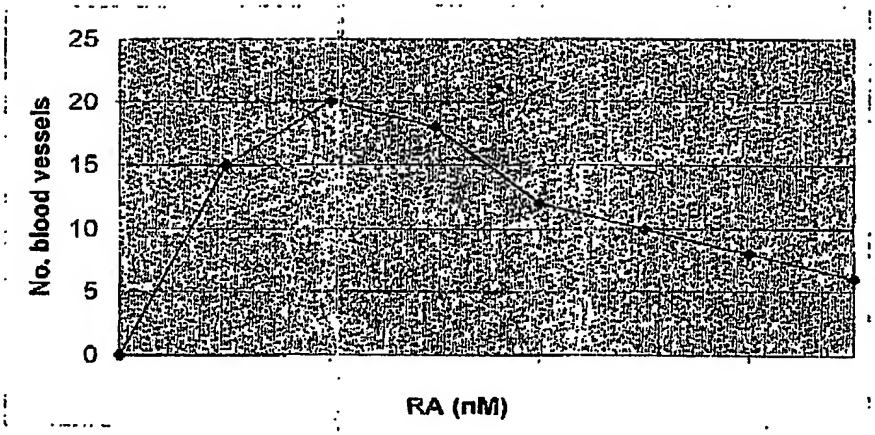


Figure 10

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